# Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli

Daniel J. Cavanaugh[a,1], Hyosang Lee[b,c,1], Liching Lo[b,c,1], Shannon D. Shields[a,1], Mark J. Zylka[d], Allan I. Basbaum[a,2], and David J. Anderson[b,c,2]

[a]Department of Anatomy, University of California, San Francisco CA 94158; [b]Division of Biology 216-76 and [c]Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125; and [d]Department of Cell and Molecular Physiology, University of North Carolina, Chapel Hill, NC 27599

Contributed by David J. Anderson, February 12, 2009 (sent for review December 22, 2008)

**Behavioral responses to painful stimuli require peripheral sensory neurons called nociceptors. Electrophysiological studies show that most C-fiber nociceptors are polymodal (i.e., respond to multiple noxious stimulus modalities, such as mechanical and thermal); nevertheless, these stimuli are perceived as distinct. Therefore, it is believed that discrimination among these modalities only occurs at spinal or supraspinal levels of processing. Here, we provide evidence to the contrary. Genetic ablation in adulthood of unmyelinated sensory neurons expressing the G protein-coupled receptor Mrgprd reduces behavioral sensitivity to noxious mechanical stimuli but not to heat or cold stimuli. Conversely, pharmacological ablation of the central branches of TRPV1$^+$ nociceptors, which constitute a nonoverlapping population, selectively abolishes noxious heat pain sensitivity. Combined elimination of both populations yielded an additive phenotype with no additional behavioral deficits, ruling out a redundant contribution of these populations to heat and mechanical pain sensitivity. This double-dissociation suggests that the brain can distinguish different noxious stimulus modalities from the earliest stages of sensory processing.**

Mrgprd | nociception | TRPV1

Nociceptors are heterogeneous by a variety of molecular criteria (1–4), but the functional significance of this heterogeneity is unclear. Electrophysiological recordings of unmyelinated primary afferent (C) fibers from dorsal root ganglia (DRG) show that most (>70%) nociceptors are polymodal: they can be activated by multiple types of painful stimuli, such as mechanical or thermal (5–7). This has led to a prevailing view that the brain's ability to discriminate different noxious stimulus modalities is unlikely attributable to modality-specific primary nociceptor subsets. Rather, it is believed that modality discrimination occurs by the decoding of nociceptor inputs in higher order spinal cord or brain areas (8, 9). A prediction of this hypothesis is that targeted ablation of any single specific nociceptor subpopulation should cause deficits in behavioral responses to noxious stimuli of multiple modalities. Indeed, some previous studies using genetic- or immunotoxin-based methods support this prediction (10–12).

A critical issue in such experiments, however, regards the cellular specificity and timing of administration of the markers/reagents used for ablation. For example, if ablation of a population of DRG neurons using a given marker caused deficits in behavioral sensitivity to multiple pain modalities (10–12), it could reflect the expression of this marker either in a homogeneous population of polymodal nociceptors or in multiple modality-specific subpopulations. Modality specificity of nociceptor subpopulations could also be difficult to detect if ablation is performed constitutively using markers that, although specific in adulthood, are transiently expressed more broadly during development (13).

We have examined the behavioral consequences of selectively eliminating 2 nonoverlapping subsets of nociceptors, based on their expression of specific receptors, in the adult. Genetic ablation of neurons that express the sensory neuron-specific G protein-coupled receptor Mrgprd (2, 14) caused specific deficits in the behavioral response to noxious mechanical stimuli but not to noxious heat or cold stimuli. Conversely, pharmacological ablation of the central projections of neurons that express the heat-sensitive channel TRPV1 (15) caused a complete loss of heat pain sensitivity, without affecting responses to noxious mechanical or cold stimuli. Combined elimination of both populations yielded an additive phenotype with no further behavioral deficits. These data reveal the existence of distinct subsets of primary sensory neurons that selectively mediate behavioral responses to different noxious stimulus modalities.

## Results

**Conditional Ablation of Mrgprd$^+$ Nociceptors.** Mrgprd$^+$ afferents exclusively innervate the epidermis and constitute >90% of all nonpeptidergic cutaneous C-fibers (2, 14, 16). These neurons bind isolectin IB4 and terminate in inner lamina II of the spinal cord dorsal horn (4). In vitro, Mrgprd$^+$ neurons exhibit electrophysiological properties characteristic of nociceptors (17) and behave as C-polymodal units in ex vivo recordings (K. K. Rau, S. L. McIlwrath, H. Wang, J. J. Lawson, M. P. Jankowski, M.J.Z., D.J.A., H. R. Koerber, unpublished data).

To determine the behavioral consequences of ablating Mrgprd$^+$ neurons, we used a conditional strategy (18, 19), in which the human diphtheria toxin receptor (DTR) was inserted in the *Mrgprd* locus (Fig. 1*A*) by homologous recombination in murine embryonic stem cells. The human DTR binds diphtheria toxin (DTX) with $10^5$-fold higher affinity than does the endogenous mouse receptor. Heterozygous *Mrgprd$^{DTR/+}$* mice (hereafter referred to as *Mrgprd$^{DTR}$* mice) expressed DTR in an identical pattern as a GFP reporter expressed from a second targeted *Mrgprd* allele (Fig. 1 *B–D*). Injection of DTX into adult *Mrgprd$^{DTR}$* mice produced a virtually complete (>98%) loss of Mrgprd$^+$ cell bodies in the DRG (Fig. 1 *E* and *F*) and in their central and peripheral fibers (Fig. 1 *I–N*). Consistent with Mrgprd expression in the nonpeptidergic afferents, DTX treatment caused an 82.4% reduction in labeling for IB4 but no change in labeling for calcitonin gene-related peptide (CGRP), a marker of peptidergic afferents [Fig. 1 *E–N* and supporting information (SI) Table S1]. The overall reduction in neuron number is commensurate with the size of the Mrgprd$^+$ popula-

Author contributions: D.J.C., H.L., S.D.S., M.J.Z., A.I.B., and D.J.A. designed research; D.J.C., H.L., L.L., S.D.S., and M.J.Z. performed research; D.J.C., H.L., L.L., S.D.S., M.J.Z., A.I.B., and D.J.A. analyzed data; and D.J.C., A.I.B., and D.J.A. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

[1]D.J.C., H.L., L.L., and S.D.S. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: allan.basbaum@ucsf.edu or wuwei@caltech.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0901507106/DCSupplemental.

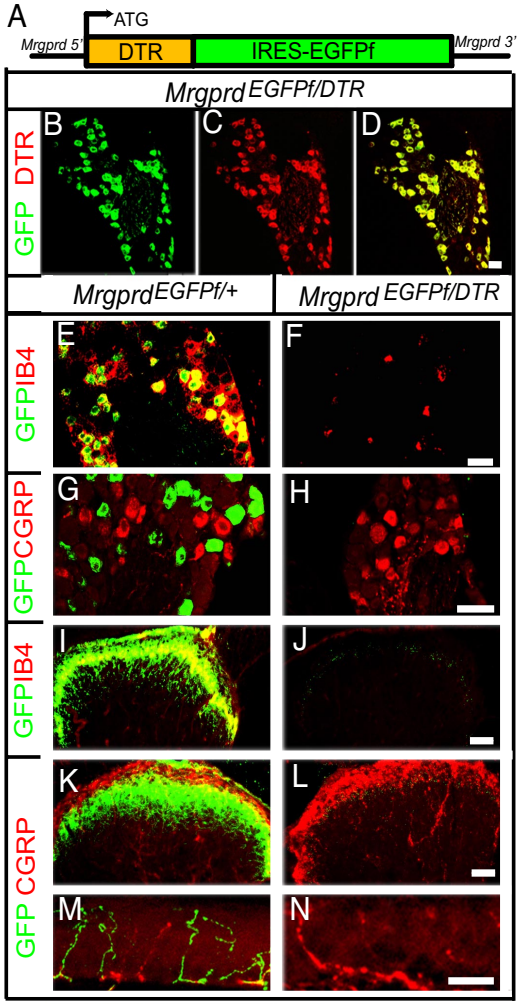


**Fig. 1.** Specific ablation of Mrgprd$^+$ neurons in mice. (*A*) *Mrgprd$^{DTR}$* targeting construct. (*B–D*) DTR expression in the absence of DTX does not impair the survival of Mrgprd$^+$ neurons, determined using an independent farnesylated enhanced green fluorescent protein (EGFPf)-expressing allele in *Mrgprd$^{EGFPf/DTR}$* transheterozygous mice. Sections of the DRG (*E–H*), spinal cord (*I–L*), and glabrous skin (*M* and *N*) from DTX-treated *Mrgprd$^{EGFPf/+}$* mice (*E*, *G*, *I*, *K*, and *M*) and *Mrgprd$^{EGFPf/DTR}$* mice (*F*, *H*, *J*, *L*, and *N*) were stained for the indicated markers. (*F*, *H*, *J*, *L*, and *N*). Note selective loss of Mrgprd$^+$ cells (green) and fibers. See Table S1 for quantification. (Scale bar in *D*, *F*, *H*, *J*, *L*: 50 $\mu$m; scale bar in *N*: 25 $\mu$m.)

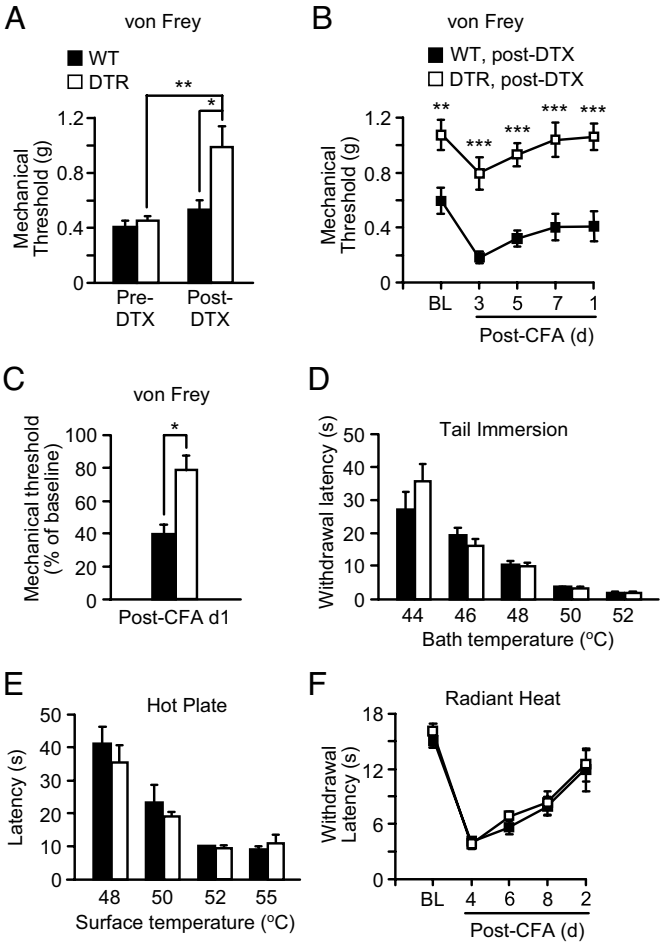


**Fig. 2.** Mice lacking Mrgprd$^+$ neurons exhibit selective deficits in mechanosensitivity. (*A*) Mechanical thresholds determined with the von Frey test before and after DTX injection (Student's *t* test: *, $P < 0.05$, **, $P < 0.01$). (*B*) von Frey test before (BL) and after CFA injection (two-way ANOVA with Bonferroni posttests: **, $P < 0.01$, ***, $P < 0.001$). (*C*) Normalized mechanical threshold at post-CFA day 1 relative to pre-CFA baseline (Mann-Whitney *U* test: *, $P < 0.05$). (*D*) Tail immersion test. (*E*) Hot plate test. The 55 °C data used a separate cohort of mice. (*F*) Radiant heat test before (BL) and after CFA injection. Data represent means ± SEM; $n = 6–10$ for all tests.

tion, suggesting that no other population is affected. No loss of Mrgprd$^+$ neurons in the DRG was observed in mice without DTX injection (Fig. 1 *B–D* and Table S1). *Mrgprd$^{DTR}$* mice were normal in viability, overall appearance, and body weight both before and after DTX treatment.

**Selective Reduction of Behavioral Responses to Noxious Mechanical Stimuli Following Ablation of Mrgprd$^+$ Neurons.** To determine the behavioral consequences of ablating Mrgprd$^+$ neurons, we measured paw withdrawal responses to calibrated von Frey filaments. Before DTX treatment, the mechanical threshold of *Mrgprd$^{DTR}$* mice was not different from that of WT littermates (Fig. 2*A*). Following DTX, however, the mechanical threshold of *Mrgprd$^{DTR}$* mice more than doubled (from 0.41 ± 0.04 g to 0.99 ± 0.15 g; Fig. 2*A*; $P < 0.01$). There was no significant change in WT mice after DTX treatment (Fig. 2*A*). The elevated mechanical threshold in *Mrgprd$^{DTR}$* mice persisted for at least 31 days after DTX (not shown). The response frequency to a series of von Frey filaments ranging from 0.16–1.4 g was also significantly reduced in DTX-treated *Mrgprd$^{DTR}$* mice relative to controls (Fig. S1). These data demonstrate that Mrgprd$^+$ neurons are necessary for normal responsiveness to acute noxious mechanical stimuli. Importantly, no deficits in mechanical pain sensitivity were detectable in mice in which Mrgprd$^+$ neurons were deleted from birth (Fig. S2 and Fig. S3), suggesting that other nociceptor populations can functionally compensate for the loss of Mrgprd$^+$ neurons, but only if the loss occurs sufficiently early in development (18).

We next asked whether Mrgprd$^+$ afferents also contribute to the mechanical hypersensitivity caused by hind paw injection of an inflammatory mediator, complete Freund's adjuvant (CFA). The mechanical thresholds of DTX-treated *Mrgprd$^{DTR}$* mice were significantly higher than those of DTX-treated WT mice at all time points following CFA injection (Fig. 2*B*; $P < 0.01$). In control (DTX-treated WT) mice, mechanical thresholds dropped significantly 1 day following CFA, to 40% of baseline (Fig. 2*C*; $P < 0.05$) and recovered to pre-CFA baseline by day 3 (Fig. 2*B*; $P < 0.01$). In contrast, the mechanical threshold in DTX-treated *Mrgprd$^{DTR}$* mice at 1 day after CFA treatment was only reduced to 80% of pre-CFA values. Thus, Mrgprd$^+$ neurons

are required for full expression of mechanical hypersensitivity after tissue injury.

**Behavioral Responses to Noxious Heat and Cold Stimuli Are Normal in DTX-Treated *Mrgprd$^{DTR}$* Mice.** Strikingly, DTX-treated *Mrgprd$^{DTR}$* mice exhibited no deficits in their behavioral sensitivity to noxious heat, as scored by tail withdrawal latency from a hot-water bath (Fig. 2*D*), latency to exhibit evidence of discomfort (paw shaking and licking) on a hot plate (Fig. 2*E*), or latency to paw withdrawal from radiant heat (Fig. 2*F*, BL). Following CFA injection, DTX-treated *Mrgprd$^{DTR}$* and WT mice developed equivalent heat hypersensitivity (Fig. 2*F*). Thus, Mrgprd$^+$ neurons are dispensable for baseline heat pain sensitivity as well as for CFA-induced sensitization to heat pain in vivo.

We next evaluated cold sensitivity in the DTX-treated *Mrgprd$^{DTR}$* mice. In a test of temperature preference between a 32 °C chamber and a chamber at variable cold temperatures (20 to 5 °C), both DTX-treated WT and *Mrgprd$^{DTR}$* mice preferred the 32 °C chamber (Fig. S4*A*). Furthermore, DTX-treated WT and *Mrgprd$^{DTR}$* mice exhibited identical paw withdrawal latencies on a −5 °C plate (Fig. S4*B*). Taken together, these results indicate that Mrgprd$^+$ neurons are not required for normal cold sensitivity.

The lack of a requirement for Mrgprd$^+$ neurons in behavioral responses to noxious heat was surprising, given their polymodal properties. To investigate whether ablation of Mrgprd$^+$ neurons might be compensated for by other heat-sensitive nociceptor populations, we sought to ablate a known heat-sensitive C-fiber population, while sparing Mrgprd$^+$ neurons. We therefore focused on the large population of TRPV1$^+$ nociceptors (20). Previous studies indicated that pharmacological ablation of TRPV1$^+$ afferents causes at least a partial loss of heat pain sensitivity (21–23). Furthermore, less than 10% of Mrgprd$^+$ neurons express TRPV1 in vivo (2, 14), and no more than 10% respond to the TRPV1 agonist capsaicin in vitro (17). TRPV1$^+$ and Mrgprd$^+$ neurons are therefore largely nonoverlapping populations.

**Ablation of the Central Terminals of TRPV1$^+$ Afferents.** Because TRPV1 is also expressed in cells outside of the DRG (24), we could not use the DTR strategy to eliminate TRPV1$^+$ nociceptors selectively. Instead, we exploited the fact that high doses of capsaicin destroy TRPV1$^+$ fibers. Intrathecal injection of capsaicin eliminated TRPV1$^+$ afferent fibers in the lumbar spinal cord (Fig. 3 *A* and *E*); however, TRPV1 staining of DRG cell bodies was preserved (Fig. S5 *A–D*). Importantly, capsaicin injection also eliminated retrograde transport of Fluorogold (Fluorochrome, LLC) from the spinal cord to the cell bodies of TRPV1$^+$ neurons (Fig. S5 *A–C*), indicating that the loss of TRPV1 fiber staining reflected destruction of the central terminals of the TRPV1$^+$ nociceptors and not simply down-regulation of TRPV1 expression.

The pharmacological ablation of TRPV1$^+$ afferents spared the Mrgprd$^+$ afferent population, as revealed by the preserved expression of a GFP reporter included in the DTR targeting cassette (Figs. 1*A* and 3*F*). Consistent with this, IB4 binding was unchanged (Fig. 3 *C* and *E*). In contrast, there was a significant reduction in the expression of CGRP, which is found in many TRPV1$^+$ neurons, to 54.5 ± 5.0% of that of vehicle-treated controls ($P = 0.0004$; Fig. 3 *B* and *E*). Immunoreactivity for other markers of TRPV1 afferents was also reduced in the spinal cord [i.e., the 5HT1D subtype of serotonin receptor (25) and the water channel aquaporin 1 (26); Fig. S6 *A*, *B*, and *E*]. Importantly, we found no change in immunoreactivity for the substance P receptor (NK1), a marker of spinal cord lamina I projection neurons that are postsynaptic targets of TRPV1$^+$ afferents, or for calbindin, which marks a large population of spinal cord interneurons (Fig. S6 *C–E*). These observations indicate that capsaicin treatment did not produce a generalized neurotoxic effect in the spinal cord.

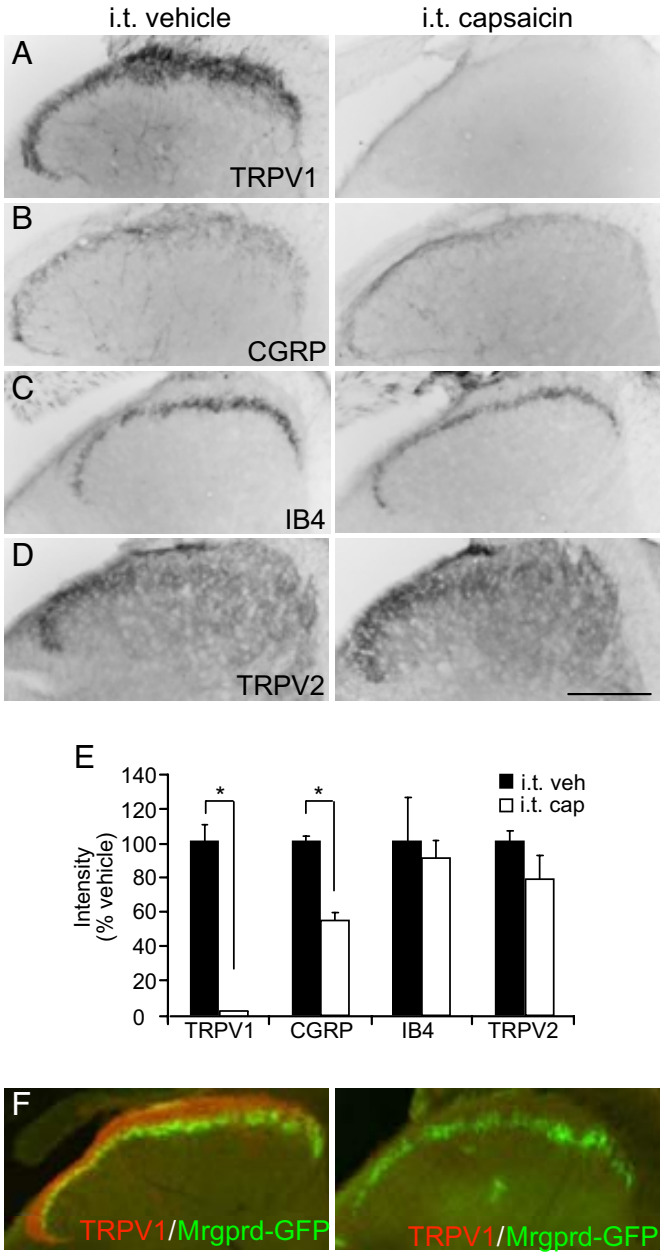


**Fig. 3.** Intrathecal (i.t.) capsaicin treatment selectively ablates the central terminals of TRPV1$^+$ nociceptors. (*A–D*) Immunostaining in lumbar dorsal horn 16 days after i.t. injection of vehicle (*Left*) or capsaicin (*Right*). Staining for TRPV1 (*A*) and CGRP (*B*) was significantly reduced by capsaicin. Staining for IB4 (*C*) and TRPV2 (*D*) was unchanged. (*E*) Density of staining: mean ± SEM (*, $P < 0.0005$, Student's *t* test). For TRPV1 staining, $n = 8$ for capsaicin-treated mice, $n = 10$ for vehicle-treated mice, and $n = 4$ per group for all others. (*F*) Double-labeling for TRPV1 (red) and Mrgprd-GFP (green) in vehicle- (*Left*) and capsaicin-treated (*Right*) mice. (Scale bar: 200 μm.)

**Intrathecal Capsaicin Treatment Eliminates Behavioral Responses to Noxious Heat.** Capsaicin-treated mice showed a complete and prolonged behavioral insensitivity to heat (Fig. 4 *A* and *B*). When tested on a 55 °C hot plate, vehicle-treated mice licked their hind paw with a latency of 11.8 ± 0.8 s, whereas capsaicin-treated mice were unresponsive up to the 30-s cutoff ($P < 0.0001$). Consistent with these behavioral data, capsaicin treatment eliminated induction of Fos, a marker of neuronal activity, in the dorsal horn of the spinal cord following hind paw exposure to a 55 °C stimulus (Fig. 4 *E* and *F*). Capsaicin-treated mice also showed no withdrawal of

NEUROSCIENCE

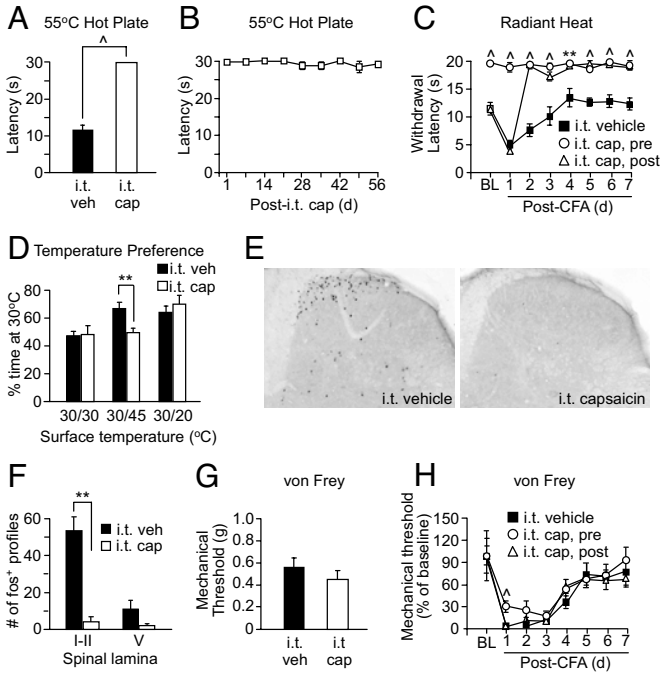


**Fig. 4.** Capsaicin (cap)-treated mice exhibit a complete and selective loss of heat pain sensitivity. Latency to lick or jump on 55 °C hot plate 1 day after intrathecal (i.t.) injection of cap or vehicle (veh) ($\hat{P} < 0.0001$, Student's *t* test) (*A*) and at weekly intervals after injection of cap ($n = 4$) (*B*). (*C*) CFA-induced heat hypersensitivity (**, $P < 0.01$, $\hat{P} < 0.0001$, two-way repeated measures ANOVA with Bonferroni posttest). (*D*) Temperature preference assay (**, $P < 0.01$, Student's *t* test). (*E*) Fos induced by hind paw immersion in 55 °C water bath. (*F*) Fos$^+$ neurons in laminae I/II and V per 40-$\mu$m section (**, $P < 0.01$, Student's *t* test; $n = 3$ per group). (*G*) Mechanical threshold (von Frey test). (*H*) CFA-induced mechanical hypersensitivity ($\hat{P} < 0.0001$, two-way repeated measures ANOVA with Bonferroni posttest). Data represent mean ± SEM. Unless otherwise noted, $n = 8$–10.

the hind paw in response to radiant heating ($P < 0.0001$; Fig. 4*C*, BL), and did not discriminate between 30 and 45 °C in the temperature preference assay, whereas control mice strongly preferred 30 °C (Fig. 4*D*; $P = 0.008$).

TRPV1$^+$ fiber ablation also affected both the induction and maintenance of behavioral heat hypersensitivity following CFA injection. In vehicle-treated mice, CFA injection produced a profound heat hypersensitivity that returned to baseline over the course of 3 days. In contrast, mice pretreated with capsaicin 1 week before CFA injection were not only unresponsive to heat before the inflammation was induced but showed no change in sensitivity following CFA injection (Fig. 4*C*). Even when mice were treated with capsaicin 1 day after CFA injection, they completely lost sensitivity to noxious heat ($P < 0.0001$). Thus, ablation of TRPV1$^+$ fibers abolishes all heat pain sensitivity under normal conditions and in the setting of injury.

The complete loss of heat responsiveness following intrathecal capsaicin indicates that Mrgprd$^+$ neurons are unable to compensate for the absence of TRPV1$^+$ afferents. These results also indicate that other heat-sensitive nociceptors, such as those that express the capsaicin-insensitive heat channel TRPV2 (27, 28), cannot compensate either. Capsaicin-treatment caused no change in TRPV2 staining ($P = 0.21$; Fig. 3 *D* and *E*), confirming that TRPV2$^+$ neurons are spared by this manipulation.

**Behavioral Responses to Mechanical and Cold Stimuli Are Normal in Capsaicin-Treated Mice.** Capsaicin treatment did not influence behavioral responses to either mechanical or cold stimuli. The mechanical withdrawal threshold in capsaicin-treated mice did not differ from that of vehicle-treated mice ($P = 0.47$; Fig. 4*G*). Furthermore, CFA-induced mechanical hypersensitivity persisted in mice treated with capsaicin either before or after CFA injection (Fig. 4*H*). Although treatment with capsaicin before CFA injection caused a slight reduction in mechanical hypersensitivity at 1 day after CFA injection (Fig. 4*H*; $P = 0.0001$), treatment with capsaicin after CFA injection had no effect on mechanical hypersensitivity. Thus, TRPV1$^+$ afferents are not required for mechanical hypersensitivity following injury but may facilitate the initial development of hypersensitivity, perhaps by modulating other populations of mechanoreceptive neurons.

Capsaicin-treated mice also exhibited normal cold pain sensitivity, as assessed by hind paw withdrawal latency on a −5 °C plate (Fig. S4*C*) and discrimination between 30 and 20 °C in the temperature preference test (Fig. 4*D*). Thus, the perception of both moderate and intense cold as aversive/painful is preserved in the absence of input carried by TRPV1$^+$ nociceptors. Because the TRPA1 channel is found in a subset of TRPV1 afferents (29), this result is consistent with our conclusion that TRPA1 is not required for acute cold-evoked pain behavior (30).

**Combined Ablation of Mrgprd and TRPV1-Expressing Nociceptors Does Not Produce Further Deficits.** TRPV1$^+$ afferents were not sufficient to support full mechanical sensitivity in the absence of Mrgprd$^+$ neurons. Nevertheless, the partial loss of mechanical pain sensitivity in DTX-treated *Mrgprd$^{DTR}$* mice left open the possibility that TRPV1$^+$ afferents contribute to the residual mechanical sensitivity in these mice. To address this possibility, we compared mechanical sensitivity in DTX-treated *Mrgprd$^{DTR}$* mice before and after ablation of TRPV1$^+$ afferents.

Importantly, capsaicin treatment in mice lacking Mrgprd$^+$ neurons produced no further decrease in mechanosensitivity compared with that observed before capsaicin treatment (Fig. 5*B*; 1.05 ± 0.38 g before and 0.87 ± 0.22 g after capsaicin treatment; $P = 0.315$). As expected, the heat sensitivity of DTX-treated *Mrgprd$^{DTR}$* mice was fully eliminated following capsaicin treatment (Fig. 5*A; P* < 0.001), confirming the efficacy of TRPV1$^+$ fiber ablation. Thus, TRPV1$^+$ neurons do not contribute to the residual mechanical pain sensitivity in *Mrgprd$^{DTR}$*/DTX mice. This residual sensitivity must therefore reflect a contribution of the remaining TRPV1$^-$ and Mrgprd$^-$ cutaneous C fibers and/or of myelinated afferents (e.g., high-threshold mechanosensitive Aδ fibers).

## Discussion

Genetic approaches to nociception have focused primarily on identifying individual molecules that transduce painful stimuli. However, the discrimination of different pain modalities by the brain depends not only on which molecules are activated but on which neurons are activated. Primary afferent nociceptors are heterogeneous; therefore, an understanding of the behavioral function of different subsets of these neurons is essential to deciphering the logic by which different types of painful stimuli are sensed and encoded. Here, we selectively ablated 2 nonoverlapping populations of nociceptors and observed a double dissociation between noxious mechanical and heat pain sensitivity. These data suggest that behavioral discrimination between different pain modalities can occur at the earliest stages of sensory processing.

A caveat is that our behavioral observations are constrained by test cutoff and intensity limitations that are necessary to prevent tissue damage. Therefore, we cannot exclude that these nociceptor classes contribute to behavioral responses to both heat and mechanical stimuli, but only at stimulus intensities greater than those we tested. Furthermore, because mechanical pain sensitivity is only partially reduced in mice lacking Mrgprd$^+$ neurons, our results do not rule out the existence of Mrgprd$^-$ subpopulations of mechanosensitive nociceptors that also mediate behavioral responses to other noxious stimulus modalities.

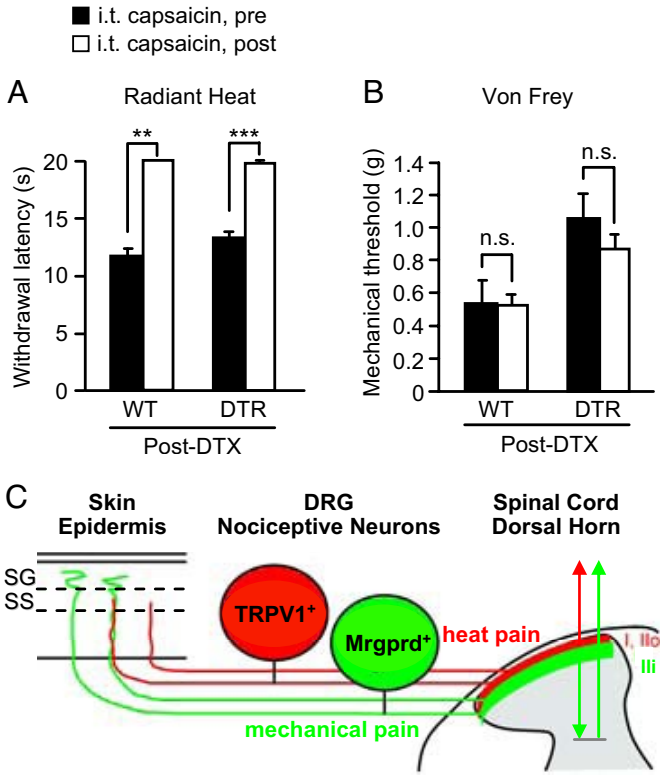


**Fig. 5.** TRPV1$^+$ neurons do not contribute to residual mechanical pain sensitivity in mice lacking Mrgprd$^+$ neurons. Mechanical (von Frey test) (*A*) and heat (radiant heat) (*B*) responsiveness in DTX-treated WT or *Mrgprd*$^{DTR}$ mice before and after intrathecal injection of capsaicin ($n = 3$ for WT/DTX and $n = 6$ for *Mrgprd*$^{DTR}$/DTX; **, $P < 0.001$, ***, $P < 0.0001$; n.s., not significant; Student's *t* test). (*C*) Schematic illustrating modality-specific contributions of Mrgprd$^+$ and TRPV1$^+$ fibers to behavior and their termination zones in the epidermis and spinal cord (14). The second-order targets of Mrgprd$^+$ afferents are shown projecting to deeper laminae before engaging ascending pathways (1). SG, stratum granulosum; SS, stratum spinosum; I, lamina I; IIo, lamina II outer; IIi, lamina II inner.

**Mrgprd$^+$ Neurons Are Selectively Required for Painful Mechanosensation.** Our data indicate that Mrgprd$^+$ neurons are necessary for full behavioral sensitivity to noxious mechanical but not thermal stimuli. The lack of a heat pain deficit in mice lacking these neurons cannot be explained by either redundancy or compensation, because all heat-pain sensitivity is lost in mice that lack TRPV1$^+$ afferents but retain nearly all Mrgprd$^+$ neurons. Nevertheless, it is possible that Mrgprd$^+$ neurons contribute to heat pain sensitivity in intact animals in a manner dependent on TRPV1$^+$ neurons.

Previous studies have reported that ablation of IB4$^+$ neurons (which include all Mrgprd$^+$ neurons) using an IB4-saporin conjugate transiently reduced both mechanical and heat pain sensitivity (11, 12), in contrast to the selective and prolonged mechanosensitive deficit in mice lacking Mrgprd$^+$ neurons. However, IB4-saporin targets a carbohydrate epitope present on multiple cell types, whereas Mrgprd is exclusively expressed in unmyelinated afferents (2, 14). Moreover, the IB4-saporin experiments were performed in the rat, in which IB4 labels a more heterogeneous population of neurons than does Mrgprd in the mouse (31). Therefore, the cellular specificity afforded by targeted ablation of Mrgprd$^+$ neurons in the mouse is much greater than that achieved by ablation of IB4$^+$ neurons in the rat. Whether different species of rodents exhibit different degrees of nociceptor specialization is an interesting question for future investigation. The recent observation that pharmacological manipulation of TRPV1 channels in the rat affects mechanical as well as heat sensitivity (32) suggests either that the neurons expressing these channels include mechanosensitive nociceptors in that species or that such nociceptors can be non-cell autonomously influenced by manipulation of heat-sensitive nociceptors.

Abrahamsen et al. (10) recently reported that genetic ablation of nociceptors expressing the Nav1.8 sodium channel, which include most IB4$^+$ neurons, caused profound deficits in basal mechanical and cold pain sensitivity as well as inflammatory pain deficits. In contrast, ablation of Mrgprd$^+$ neurons (which constitute ≈90% of IB4$^+$ cutaneous afferents) impaired mechanical but not cold pain sensitivity. It is likely that elimination of Nav1.8$^+$ DRG neurons leads to changes in the behavioral response to multiple modalities, because this channel is present in >85% of small-diameter neurons, including both Mrgprd$^+$ and TRPV1$^+$ neurons. By contrast, the manipulations used in the present study target more specific populations of neurons and reveal more specific phenotypes.

**TRPV1$^+$ Neurons Are Essential for Heat-Pain Sensitivity.** Ablation of TRPV1$^+$ central afferent fibers by capsaicin injection abolished heat-pain sensitivity, without affecting the responses to noxious mechanical or cold stimulation. Previous studies using similar approaches reported less complete deficits in heat pain sensitivity than we observed here, but the effects were also modality specific (21–23, 33, 34). The partial behavioral deficits observed in earlier studies using capsaicin or resiniferatoxin treatment likely reflected incomplete ablation of the TRPV1$^+$ afferent fibers, because these studies were performed before the availability of reagents to monitor TRPV1 expression.

It is surprising, given our results, that Abrahamsen et al. (10) observed only subtle deficits in heat pain sensitivity following ablation of Nav1.8$^+$ neurons, because this manipulation destroyed a large fraction of TRPV1$^+$ neurons. This may reflect compensation by the small fraction of TRPV1$^+$ neurons that were spared. Alternatively, the fact that Nav1.8$^+$ neurons were constitutively ablated from the embryonic stage at which this gene is first transcribed (10) could permit compensation by other neuronal populations during development and maturation, as we observed following constitutive ablation of Mrpgrd$^+$ neurons (Fig. S2 and Fig. S3).

The complete heat pain insensitivity of mice lacking central TRPV1$^+$ fibers contrasts with the phenotype of *Trpv1* knockout mice, which exhibit only a partial reduction in heat sensitivity (35, 36). The residual heat pain behavior in these gene knockout mice must therefore reflect the existence of additional molecular heat transducers that act, either cell autonomously or nonautonomously, via TRPV1$^+$ neurons.

**Physiology vs. Behavior.** How do we reconcile our behavioral observations with the fact that the majority of C fibers are polymodal by electrophysiological criteria (6)? It is possible that the response properties of these nociceptors, as determined by ex vivo electrophysiological recordings, differ from those exhibited by these neurons in vivo. Importantly perhaps, recordings from identified Mrgprd$^+$ and TRPV1$^+$ afferents have been performed on hairy skin, whereas our behavioral assays are performed using stimuli applied to glabrous skin. Conceivably, the properties of these C-fibers in glabrous skin more closely correlate with our behavioral finding of modality specificity. Alternatively, Mrgprd$^+$ and TRPV1$^+$ neurons may indeed be activated by both heat and mechanical stimuli in vivo, but these 2 types of stimulus modalities may evoke different spiking patterns within each class of nociceptors (e.g., ref. 37). If so, perhaps only a single "preferred" modality is able to activate each nociceptor subtype to a level sufficient to drive second-order spinal cord neurons above a threshold required to evoke nocifensive behavior. It is also possible that these nociceptor subtypes convey polymodal information to the spinal cord but that this information contributes to aspects of the pain experience that are not measurable by the behavioral assays we used. Electrophysiological recordings from spinal cord neurons of mice

lacking Mrgprd$^+$ or TRPV1$^+$ afferents should help to resolve these questions. Finally, although polymodal nociceptors predominate, DRG neurons that are modality specific by electrophysiological criteria do exist. For example, a recent electrophysiological study described a heat-selective subpopulation of nociceptors, all of which expressed TRPV1 (6). Our data suggest that this population is likely to be particularly relevant for heat-evoked behavioral responses.

TRPV1$^+$ and Mrgprd$^+$/IB4 fibers target distinct laminae in the dorsal horn of the spinal cord (4), innervate different layers of the epidermis (14), and likely engage distinct ascending circuits (1) (Fig. 5*C*). These parallel pathways thus represent a neuroanatomical substrate for the behaviorally relevant processing of different pain modalities by these 2 classes of peripheral nociceptors. Whether these pathways exclusively mediate mechanical vs. heat pain discrimination or have additional functions is not clear. Whatever the case, our data suggest that this discrimination can be achieved at the earliest stages of nociceptive sensory processing and does not, as previously believed, exclusively emerge at spinal or supraspinal levels. Thus, as in the mammalian (38) and invertebrate (39) gustatory systems, the cellular logic of information processing in the "pain" system incorporates distinct subsets of primary sensory cells that selectively mediate appropriate behavioral responses to different stimulus modalities.

## Experimental Procedures

**Animals and Injections.** Animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the recommendations of the International Association for the Study of Pain. Two to 5 animals were housed per cage and maintained on a 12-h light/dark schedule with ad lib access to food and water. DTX (100 $\mu$g/kg) was injected i.p. on 2 days, separated by 72 h. Behavioral tests were performed 7 to 31 days after the initial DTX injection. For intrathecal capsaicin studies, adult male C57Bl6 mice (20–30 g; Charles River) were anesthetized with 1.5% isoflurane (vol/vol) and injected intrathecally with capsaicin (10 $\mu$g) or vehicle (10% ethanol (vol/vol), 10% Tween 80, saline (vol/vol)) in a volume of 5.0 $\mu$L with a luer-tipped Hamilton syringe at the level of the pelvic girdle. Behavioral tests were performed 1 to 16 days after capsaicin injection.

**Molecular Biology and Anatomy.** *Mrgprd$^{DTR}$* and *Mrgprd$^{DTA}$* targeting constructs were generated as described in *SI Materials and Methods*. Gene targeting in embryonic stem cells by homologous recombination was performed as described (14). Histology, immunohistochemistry, densitometry and c-fos analysis were performed as described in *SI Materials and Methods*.

**Behavior.** WT and *Mrgprd$^{DTR}$* mice were individually housed at least 1 week before testing, which was performed blind to genotype and treatment group during the animals' light period, as described previously (26, 40, 41). The reader is referred to *SI Materials and Methods* for a detailed description of behavioral testing.

**Statistical Analysis.** Behavioral, densitometry, and Fos data were analyzed by the Student's *t* test, one- and two-way repeated measures ANOVA (Bonferroni posttest), or the Mann-Whitney *U* test, with $P < 0.05$ considered to be significant.

**ACKNOWLEDGMENTS.** This work was supported, in part, by National Institutes of Health Grants PO1NS048499 (to D.J.A. and A.I.B.) and NS14627 (to A.I.B.); by awards from the National Alliance for Research on Schizophrenia and Depression, the Searle Scholars Program, and the Whitehall, Klingenstein, Sloan, and Rita Allen Foundations (to M.J.Z.); and by an award from the Christopher and Dana Reeve Foundation to (H.L.). We thank Kenji Kohno for the DTR (TRECK-1) cDNA clone, Joao Braz for help with retrograde tracing, Noritaka Imamachi for help with intrathecal injections, and Shirley Pease and staff for assistance with genetically modified mice. D.J.A. is an Investigator of the Howard Hughes Medical Institute.

1. Braz JM, Nassar MA, Wood JN, Basbaum AI (2005) Parallel ''pain'' pathways arise from subpopulations of primary afferent nociceptor. *Neuron* 47:787–793.
2. Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ (2001) A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell* 106:619–632.
3. Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. *Nature* 413:203–210.
4. Snider WD, McMahon SB (1998) Tackling pain at the source: New ideas about nociceptors. *Neuron* 20:629–632.
5. Cain DM, Khasabov SG, Simone DA (2001) Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: An in vivo study. *J Neurophysiol* 85:1561–1574.
6. Lawson JJ, McIlwrath SL, Woodbury CJ, Davis BM, Koerber HR (2008) TRPV1 unlike TRPV2 is restricted to a subset of mechanically insensitive cutaneous nociceptors responding to heat. *Journal of Pain* 9:298–308.
7. Perl ER (1996) Cutaneous polymodal receptors: Characteristics and plasticity. *Prog Brain Res* 113:21–37.
8. Melzack R, Wall PD (1962) On the nature of cutaneous sensory mechanisms. *Brain* 85:331–356.
9. Perl ER (2007) Ideas about pain, a historical view. *Nat Rev Neurosci* 8:71–80.
10. Abrahamsen B, et al. (2008) The cell and molecular basis of mechanical, cold, and inflammatory pain. *Science* 321:702–705.
11. Tarpley JW, Kohler MG, Martin WJ (2004) The behavioral and neuroanatomical effects of IB4-saporin treatment in rat models of nociceptive and neuropathic pain. *Brain Res* 1029:65–76.
12. Vulchanova L, et al. (2001) Cytotoxic targeting of isolectin IB4-binding sensory neurons. *Neuroscience* 108:143–155.
13. Molliver DC, Snider WD (1997) Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. *J Comp Neurol* 381:428–438.
14. Zylka MJ, Rice FL, Anderson DJ (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron* 45:17–25.
15. Caterina MJ, et al. (1997) The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature* 389:816–824.
16. Zylka MJ, Dong X, Southwell AL, Anderson DJ (2003) Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. *Proc Natl Acad Sci USA* 100:10043–10048.
17. Dussor G, Zylka MJ, Anderson DJ, McClesky EW (2008) Cutaneous sensory neurons expressing the Mrgprd receptor are putative ATP-sensing nociceptors. *J Neurophysiol* 99:1581–1589.
18. Luquet S, Perez FA, Hnasko TS, Palmiter RD (2005) NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 310:683–685.
19. Saito M, et al. (2001) Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol* 19:746–750.
20. Caterina MJ, Julius D (2001) The vanilloid receptor: A molecular gateway to the pain pathway. *Annu Rev Neurosci* 24:487–517.
21. Gamse R (1982) Capsaicin and nociception in the rat and mouse. Possible role of substance P. *Naunyn-Schmiedebergs Arch Pharmakol* 320:205–216.
22. Holzer P (1991) Capsaicin: Cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev* 43:143–201.
23. Yaksh TL, Farb DH, Leeman SE, Jessell TM (1979) Intrathecal capsaicin depletes substance P in the rat spinal cord and produces prolonged thermal analgesia. *Science* 206:481–483.
24. Caterina MJ (2003) Vanilloid receptors take a TRP beyond the sensory afferent. *Pain* 105:5–9.
25. Potrebic S, Ahn AH, Skinner K, Fields HL, Basbaum AI (2003) Peptidergic nociceptors of both trigeminal and dorsal root ganglia express serotonin 1D receptors: Implications for the selective antimigraine action of triptans. *J Neurosci* 23:10988–10997.
26. Shields SD, Mazario J, Skinner K, Basbaum AI (2007) Anatomical and functional analysis of aquaporin 1, a water channel in primary afferent neurons. *Pain* 131:8–20.
27. Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398:436–441.
28. Lewinter RD, Skinner K, Julius D, Basbaum AI (2004) Immunoreactive TRPV-2 (VRL-1), a capsaicin receptor homolog, in the spinal cord of the rat. *J Comp Neurol* 470:400–408.
29. Story GM, et al. (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 112:819–829.
30. Bautista DM, et al. (2006) TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* 124:1269–1282.
31. Price TJ, Flores CM (2007) Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *Journal of Pain* 8:263–272.
32. Binshtok AM, Bean BP, Woolf CJ (2007) Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature* 449:607–610.
33. Fitzgerald M, Woolf CJ (1982) The time course and specificity of the changes in the behavioural and dorsal horn cell responses to noxious stimuli following peripheral nerve capsaicin treatment in the rat. *Neuroscience* 7:2051–2056.
34. Karai L, et al. (2004) Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. *J Clin Invest* 113:1344–1352.
35. Caterina MJ, et al. (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288:306–313.
36. Davis JB, et al. (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 405:183–187.
37. Stucky CL, Lewin GR (1999) Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *J Neurosci* 19:6497–6505.
38. Chandrashekar J, Hoon MA, Ryba NJ, Zuker CS (2006) The receptors and cells for mammalian taste. *Nature* 444:288–294.
39. Scott K (2005) Taste recognition: Food for thought. *Neuron* 48:455–464.
40. Lee H, Iida T, Mizuno A, Suzuki M, Caterina MJ (2005) Altered thermal selection behavior in mice lacking transient receptor potential vanilloid 4. *J Neurosci* 25:1304–1310.
41. Bautista DM, et al. (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* 448:204–208.